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14. **ABSTRACT**
Considerable evidence suggests that poor prognosis in breast cancer is due to the over expression of cell surface receptor tyrosine kinases. A molecule downstream in the signaling pathways common to all these receptors is the small adaptor protein Shc. The Shc adapter protein transmits signals from the activated growth factor receptor to Ras. There are three Shc isoforms of 46, 52, and 66 kDa. The 52- and 46-kDa isoforms, which differ in their 5' initiation site, are ubiquitously expressed. Our laboratory has previously reported that most cell lines derived from breast cancers harbor constitutively tyrosine phosphorylated p46- and p52-Shc. The p66-Shc isoform, expressed through the use of an alternative promoter, contains an additional 110 amino-acid CH2 domain on its amino terminus. Recent studies have suggested that p66-Shc can act as a feedback down-regulator of growth factor signaling to Erk1/2 and c-fos, and also can act as an apoptotic sensitizer to oxidative stress. In many cell lines, the functions of p66-Shc require phosphorylation in serine-36 of its unique CH2 domain. Our laboratory has reported a strong negative correlation between the levels of tyrosine phosphorylated p52-Shc and the levels of p66-Shc in cell lines derived from human breast cancers.

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EGFR-epidermal growth factor receptor.

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INTRODUCTION

Considerable evidence suggests that poor prognosis in breast cancer is due to the over expression of cell surface receptor tyrosine kinases, such as ErbB2, IGF-1 and EGFR. A molecule downstream in the signaling pathways common to all these receptors is the small adaptor protein Shc. The Shc adapter protein transmits signals from the activated growth factor receptor to Ras.

There are three Shc isoforms of 46, 52, and 66 kDa. The 52- and 46-kDa isoforms, which differ in their 5' initiation site, are ubiquitously expressed[1]. Our laboratory has previously reported that most cell lines derived from breast cancers harbor constitutively tyrosine phosphorylated p46- and p52-Shc. The p66-Shc isoform, expressed through the use of an alternative promoter, contains an additional 110 amino-acid CH2 domain on its amino terminus. Recent studies have suggested that p66-Shc can act as a feedback down-regulator of growth factor signaling to Erk1/2 and *c-fos,* and also can act as an apoptotic sensitizer to oxidative stress[2-4]. In many cell lines, the functions of p66-Shc require phosphorylation in serine-36 of its unique CH2 domain.

Our laboratory has reported a strong negative correlation between the levels of tyrosine phosphorylated p52-Shc and the levels of p66-Shc in cell lines derived from human breast cancers[5]. This suggests the possibility that loss of p66-Shc expression confers a selective advantage for these breast cancer cells.

This research will help me to understand how p66-Shc suppresses tumorgenicity of these breast cancer cell lines by testing the hypothesis that, "*p66-Shc interferes with cell growth and tumorgenicity by downregulating key signaling pathways that regulate cell cycling, cell survival or both*".

BODY

The Body of this progress report is presented in sections according to the approved statement of work. The Specific aims and tasks for each section appear in italics.

Specific Aim 1. To determine the p66-Shc domains and post-translational modifications that are required to inhibit tumorgenicity (as measured by colony formation in soft agar).

I am employing the technique of site directed mutagenesis to make p66-Shc Ser36 mutants. I am mutating the ser-36 site to alanine and aspartate using primers of my design. After successfully obtaining these mutations, which I will confirm via sequencing, I will transfect the mutant constructs into our MDA-MB-453 and SKBR3 breast cancer cell lines. I will then grow those transfected cell lines in soft agar to determine if phosphorylation at the Ser36 site is necessary for the growth inhibitory phenotype.

utilized the tools on the stratagene website to design mutagenesis primers which would mutate he Ser-36 sequence to alanine and aspartate. To confirm the mutagenesis constructs, I sequenced the constructs. It was my goal to express the mutated sequence in the MDA-MB-453 and SKBR3 Breast cancer cells to determine if the ser-36 site is necessary for the growth inhibitory phenotype.

I was unsuccessful in obtaining a mutated sequence. My results suggest that either: A) I was unable to optimize the conditions such that my primers would prime properly, or less likely, B) that my digest to degrade starting DNA material was inefficient. My positive control makes the latter possibility much less likely.

Specific Aim 2. To elucidate the cell-biological effects of expressing p66-Shc in SKBR3 and MDA-MB-453 cells.

Task 1. Determine if p66-Shc inhibits growth and tumorgenicity by decreasing cell survival, by inhibiting passage through the cell cycle, or both.

As stated in the 2003 annual review, utilizing the methylcellulose technique has been unsuccessful in these experiments. Since then, I have tried growing the MDA-MB-453 and SKBR3 breast cancer cell lines in roller bottles and in suspension culture. Similar to our results when growing cells in the PolyHema© coated plates, the cells made cell-cell contacts and grew in both the roller bottle and suspension cultures. Our growth in PolyHema© data suggests that when cells make cell-cell contacts, they are able to overcome the growth inhibitory phenotype. Therefore, I was unable to replicate the growth inhibitory phenotype using these techniques.

I investigated the growth response of our MDA-MB-453 and SKBR3 breast cancer cell lines. It was our hypothesis that our breast cancer cells lacking p66-Shc, either, 1) lacked a "factor" which was important for cellular growth, or 2) could not respond to a factor which was important for cellular growth, due to altered growth factor receptor production or degradation. To determine whether p66-Shc non-expressing cells lacked a "factor", I utilized conditioned media from p66-Shc expressing breast cancer cells.

p66-Shc non-expressing cells did not grow in conditioned media when plated under anchorage-independent conditions. These results suggest that p66-Shc does not promote the release of a "growth factor".

Task 2. Determine subcellular localization of active (vs inactive) forms of p66-Shc and compare this to subcellular localization of p52/p46 Shc. Confocal innunofluorescence microscopy and sub-cellular fractionation will be the major approaches employed.

I planned on using the technique of cell fractionation to determine the subcellular location of p66-Shc. Because of unclear results utilizing this technique, I am working with another laboratory that is proficient in the novel approach of Quantum dot technologies to look at p66-Shc subcellular localization. Using strepavidin/biotin conjugation, and p66-Shc monoclonal antibodies made in our laboratory, I will be able to look at the subcellular localization of multiple proteins at once. This technique will also be useful in looking at protein-protein interactions as stated in Specific Aim 3, task 4.

P66-Shc protein staining 8c6

I approached this aim by utilizing direct immunofluorescence utilizing the monoclonal antibodies which were designed in our laboratory.

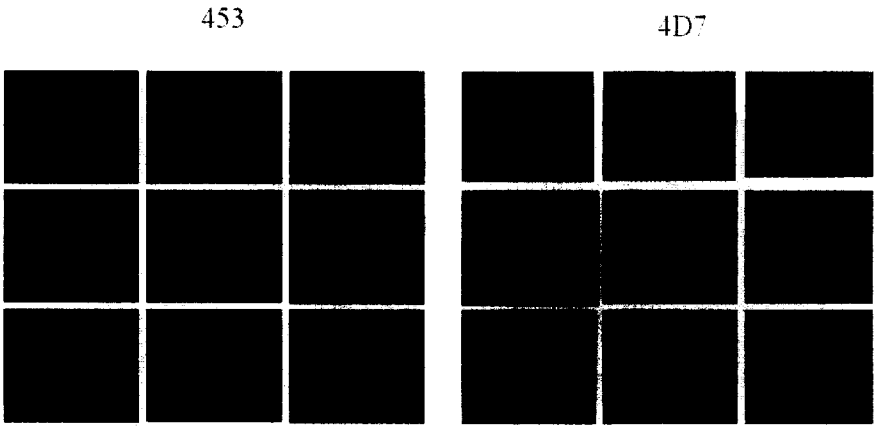


Figure 1. 453 and 4D7 cells were stained with the 2.5 ug/mL 8c6 monoclonal antibody. 1:200 anti-mouse FITC was used As a secondary antibody. Cells were counterstained with 1.5 mg/mL DAPI. Pictures were taken on a Zeiss fluorescence Microscope at 63X.

The results suggest that there is no difference in the staining pattern between the two cell lines. I repeated the same experiment utilizing the 6A8 antibody. The 6A8

monoclonal antibody gave no signal in either cell line, suggesting that A) the 6A8 antibody is not a good candidate for immuno-fluorescence, or B) that p66-Shc is not present in either cell line.

The background staining of the 8c6 antibody is high due to the fact that it recognizes linear determinants. Staining with alternative monoclonal antibodies would more conclusively show that there is no difference in the staining pattern between these two cell lines.

Specific Aim 3. To identify biochemical mechanisms whereby p66-Shc inhibits cell proliferation and tumorgenicity.

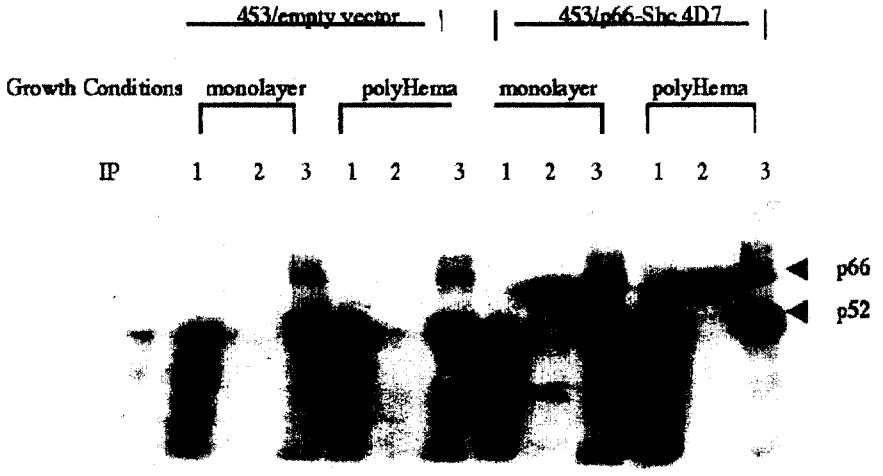
Task 3. Use of the novel "protein array chip" technology to provide leads to signaling pathways and cellular processes that have been affected by p66-Shc overexpression. Confirm by immoprecipitation.

We have not yet addressed protein interactions using the novel "protein-array" chip technology. Dr. Eugene Chin, a professor in my department, is working with this method. As stated in my grant proposal, I plan on collaborating with him on this aspect of the project. I plan to address this issue in the next year.

Task 4. Determine the effects of p66-Shc on the binding of proteins (e.g. Grb2) to endogenous p52/p46 Shc isoforms. Isolate and identify proteins (by immunoblotting and by Mass Spectrometry) that interact with active and inactive forms of p66-Shc.

Preliminarily, by co-immunoprecipitation, my data suggests that p66-Shc does not compete with p52-Shc for binding to Grb2. My data suggests that Grb2 binds p66-Shc and p52-Shc to the same extent when cells are grown under both adherent and non-adherent conditions. I have been unable to

Figure 2. The parental MDA-



immunoprecipitate Shc and stain for Grb2 because of contaminating low molecular weight immunoglobulin bands which run similarly to Grb2 in SDS-PAGE (Figure 2).

Figure 2. MB-453 cells carrying the empty pLXSN vector (453/empty vector) or the clone forced to re-express p66 Shc (453/p66-Shc 4D7) were cultured for 24 hours in serum-containing IMDM media under normal anchorage dependent conditions (monolayer) or under semi-anchorage independent conditions on polyHema-coated Petri dishes (polyHema). Proteins were extracted from the cells in a buffer containing 1% Triton-X-100, and then **immunoprecipitated with Protein A-Sepharose 4BCL beads(negative control, lanes 1)**, with **monoclonal antibody to p66 Shc (lanes 2) or with antibody to Grb2 (lanes 3)**. The immunoprecipitated proteins were resolved by 12.5% SDS PAGE, transferred to a nitrocellulose membrane, probed with a pan-Shc polyclonal antibody, and detected by ECL.

Recent data suggests that under conditions of oxidative stress, p66-Shc causes apoptosis in a number of cell lines [4]. In *p66shc*$^{-/-}$ cells the activity of the mammalian forkhead homolog, FKHRL1, is increased and forkhead inactivation is reduced. The activation of FKHRL1 allows for shuttling of the protein to the nucleus to activate the oxygen radical scavenger Catalase. Catalase functions to neutralize oxygen radicals, and therefore, inhibits apoptosis in these cells [6]. It is thought that the presence of p66-Shc induces the phosphorylation of FKHRL1 either directly or indirectly through AKT, which inactivates FKHRL1 causing the protein to remain in the nucleus. My preliminary data suggests that p66-Shc does not function through forkhead and AKT.

To confirm these conclusions, I plan to use quantum dot technology to look at protein-protein interaction and protein subcellular localization. From this, I will be able to determine the presence of p66-Shc, forkhead, Grb2 and other potential p66-Shc interacting protein in various subcellular compartments under the conditions of adherent and non-adherent growth.

Task 5. Prepare manuscript for publication and successfully write and defend my doctoral dissertation.

I have not yet prepared a manuscript for publication. I have attended a number of seminars that are required by the department of Molecular Biology, Cellular Biology and Biochemistry to fulfill requirements for the PhD degree. I have attended a number of seminars given by leaders in the field. I attended the Era of Hope conference, held in Philadelphia, PA. Additionally, I have attended and participated in the annual Molecular Biology, cellular biology and Biochemistry retreat, which includes presenting original research in a poster session. I have also met with my graduate committee in March 2005 where I updated my committee members of my progress.

I have given a seminar to biologists at the National Institutes for Standards and Technology.

I am in the process of writing and will defend in November 2005.

Specific Aim 4. To study the effects of expressing p66-Shc in SKBR3 and MDA-MB-453 cells on their tumor biology. Questions that will be addressed include: Does p66 expression inhibit tumorgenicity in mouse xenogratfs, as well as colony formation in soft agar? How is it that p66-Shc does not inhibit tumorgenicity of MCF-7 or MDA-MB-231 breast cancer cells? What is the mechanism whereby cell aggregation on polyHEMA plates seems to partially circumvent p66-Shc's ability to block anchorage independent growth?

I have not yet addressed this specific aim. I plan on addressing these issues in the coming months.

Because I was unsuccessful in my experiments, I begin looking for basic differences between the cell lines to determine whether I had lost expression in my p66-Shc expressing clone. To accomplish this goal, I looked at actin distribution and p66-Shc/pLSXN integration into the genomic DNA.

FISH analysis and restriction mapping.

MDA-MB-453 4D7

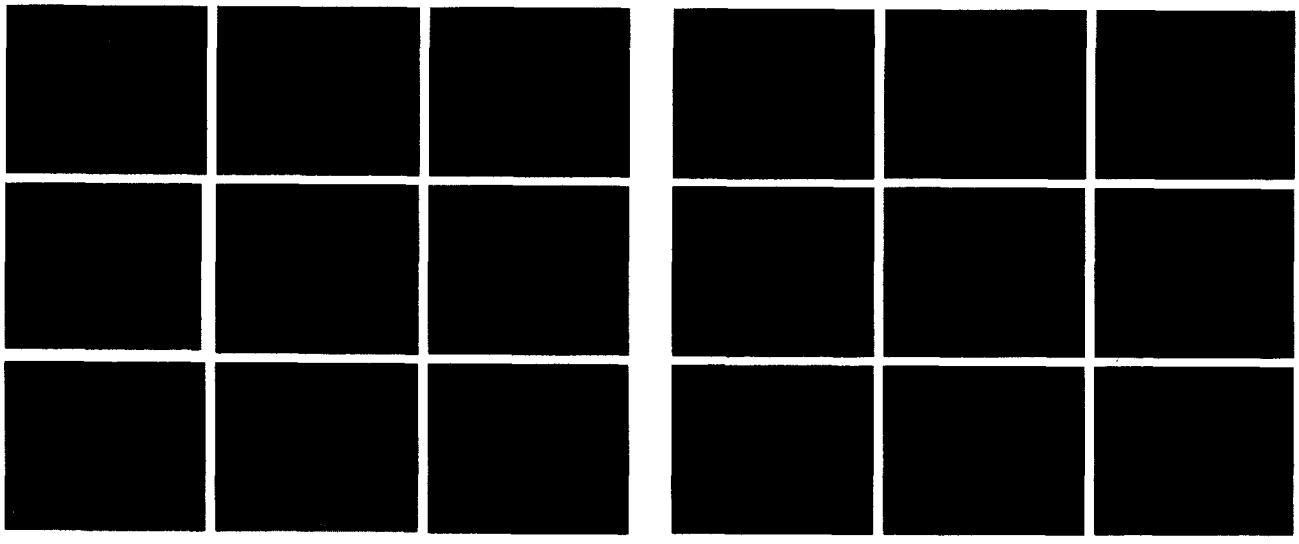


Figure 3. Images taken at 63X using the Zeiss microscope system. Exposures for Dapi = 500 msec and exposure for
FITC – 1.00 sec. 1:200 FITC. 1.5 mg/ml dapi.

I wanted to determine whether p66/pLXSN could be seen via FISH analysis (figure 3). I transfected the MDA-MB-453 cells with the p66-pLSXN construct. If the clone were stably incorporated into the genomic DNA, I would be able to obtain hybridization to a FITC labeled p66/pLSXN probe.

If hybridization were to occur, I would expect light signal on both chromatids of DNA. The results of figure 3 suggest that there is no difference in the staining pattern between the cell lines. I have repeated this experiment and come to similar conclusions.

It is possible that the p66-pLSXN that was transfected into the cells is extra-chromosomal, in which case an alternative staining pattern would be seen. My results suggest that extra-chromosomal presentation to be unlikely because I'm seeing a similar staining pattern in both cell lines. More studies would need to be performed to conclusively rule-out this possibility.

Additionally, it is difficult to see single copy genes via FISH analysis. Therefore, I employed the technique of restriction enzyme mapping (figure 4) to determine whether p66-Shc was incorporated into the genomic DNA.

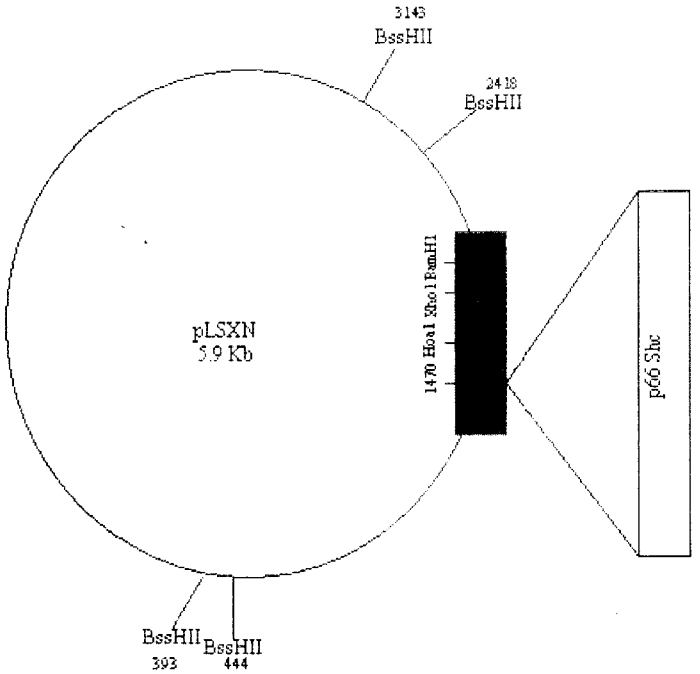


Figure 4. A restriction map of the p66-Shc/pLSXN construct. A restriction digest of genomic DNA containing the p66-Shc/pLSXN construct should create fragments of 3.9 Kb and 4.5 Kb.

I analyzed DNA integration into the chromosomes by restriction enzyme mapping with BssHII, which rarely cleaves human genomic DNA. BssHII also has two cleavage sites in the pLSXN fragment (figure 4) which would generate fragments of 3.9 and 4.5 kB.

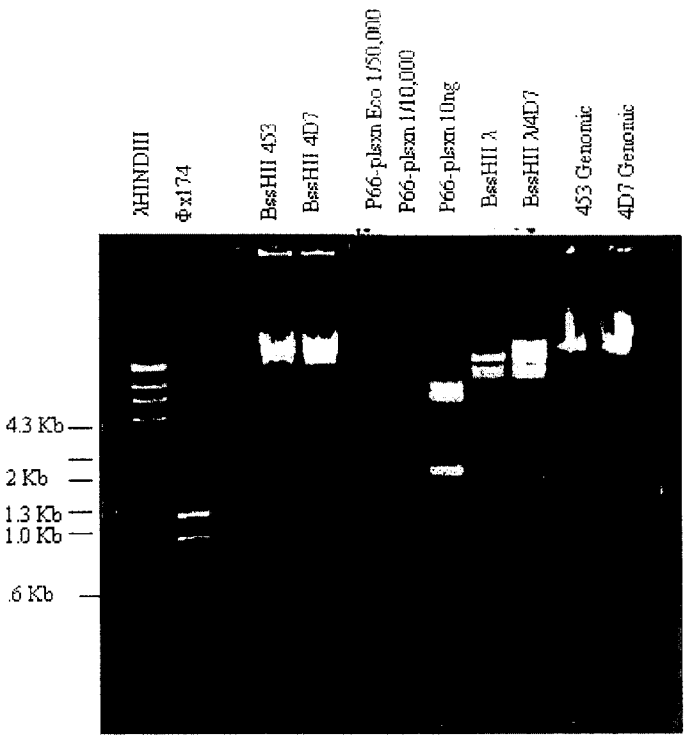


Figure 5. A BssHII restriction digest of 453 and 4D7 genomic DNA.

BssHII restriction digest experiments suggest that p66-

Shc/pLSXN construct is not present in the genomic DNA. No fragments were seen when I cleaved 453 and 4D7 cells with BssHII. Positive controls (lambda digested with BssHII) demonstrate that BssHII is functioning properly. To rule out any inhibitory activity associated with the genomic DNA preps, I included 4D7 genomic DNA in the lambda BssHII restriction digest preparations. Lambda was digested just as efficiently suggesting that there is not inhibitor activity within my genomic DNA preparations.

Southern blot analysis would be a more efficient way of looking at the restriction digests as the cleavage fragments might be lower than the detection limits of the gel.

p66-Shc regulation is a key question in understanding the mechanism of the p66-Shc negative growth phenotype. Our results suggest that p66-Shc is not regulated at the level of protein turnover. We then asked whether p66-Shc is regulated at the level of mRNA stability. I approached this question by analyzing RNA via RT-PCR analysis. I was unsuccessful in obtaining p66-Shc RNA in either 453 or the p66-Shc expressing clone.

KEY ACCOMPLISHMENTS

- Using commercially available phospho-serine p66-Shc monoclonal antibody, primary evidence suggests that p66-Shc (in MDA-MB-453) is not serine phosphorylated in cells in our cells grown under anchorage-dependent and anchorage-independent conditions. To confirm these results, I am utilizing site directed mutagenesis to mutate SER36 to alanine and aspartate.
- Using conditioned media, our evidence suggests that p66-Shc is not involved in the release of a growth factor.
- Becoming competent in the techniques of immunoflourescence and *in situ* hybridizatioin.

REPORTABLE OUTCOMES

- Poster presentation at the annual Era of Hope Breast cancer research conference.
- Seminar presentation to biologists at the National Institutes of standards and Technology.

CONCLUSIONS

Due to poor p66-Shc pSer36 monoclonal antibody availability, it is not possible to address whether p66-Shc is serine phoshporylated confidently using western blotting techniques. Therefore, I am now employing the techniques of site directed mutagenesis and sequencing to determine whether the pSer36 is necessary in the growth inhibitory phenotype. By mutating the Ser36 site to alanine and aspartate, thereby altering the sites ability to be phosphorylated, I will transfect the constructs into our breast cancer cell lines. By growing the transfected cells under anchorage independent conditions, I will be able to confidently determine whether post translational modification of the Ser36 site is important to the growth inhibitory phenotype.

Likewise, when our breast cell lines forced to express p66-Shc are grown under low O_2 conditions (hypoxic environment), p66-Shc confers a growth advantage I will also use our Ser36 mutant constructs to determine whether the Ser36 site is necessary for this unique phenotype.

In addition to the proposed work, I have begun investigating other mechanisms for the growth inhibitory phenotype. First, I am investigating the involvement of integrins in the growth inhibitory phenotype. Second, I preliminarily began investigating the role of $p21^{cip1/waf1}$ expression levels in our breast cancer cell lines. p21 has been shown to have a role in cell growth inhibition and apoptosis in HER2/NEU

overexpressing cell lines [7-9]. I am now asking questions to determine whether p21 plays a pivotal role in the inhibition of growth phenotype in breast cancer cells forced to express p66-Shc. To date, no conclusive data has come from these experiments. Third, I am investigating whether p66-Shc cells have different growth factor expression levels via the quantum dot technology.

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